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Molecular Mechanisms of Chlorhexidine Tolerance in *Burkholderia cenocepacia* Biofilms▽†

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The high tolerance of biofilm-grown *Burkholderia cepacia* complex bacteria against antimicrobial agents presents considerable problems for the treatment of infected cystic fibrosis patients and the implementation of infection control guidelines. In the present study, we analyzed the tolerance of planktonic and sessile *Burkholderia cenocepacia* J2315 cultures and examined the transcriptional response of sessile cells to treatment with chlorhexidine. At low (0.0005%) and high (0.05%) concentrations, chlorhexidine had a similar effect on both populations, but at intermediate concentrations (0.015%) the antimicrobial activity was more pronounced in planktonic cultures. The exposure of sessile cells to chlorhexidine resulted in an upregulation of the transcription of 469 (6.56%) and the downregulation of 257 (3.59%) protein-coding genes. A major group of upregulated genes in the treated biofilms encoded membrane-related and regulatory proteins. In addition, several genes coding for drug resistance determinants also were upregulated. The phenotypic analysis of RND (resistance-nodulation-division) efflux pump mutants suggests the presence of lifestyle-specific chlorhexidine tolerance mechanisms; efflux system RND-4 (BCAL2820-BCAL2822) was more responsible for chlorhexidine tolerance in planktonic cells, while other systems (RND-3 [BCAL1672-BCAL1676] and RND-9 [BCAM1945-BCAM1947]) were linked to resistance in sessile cells. After sessile cell exposure, multiple genes encoding chemotaxis and motility-related proteins were upregulated in concert with the downregulation of an adhesin-encoding gene (BCAM2143), suggesting that sessile cells tried to escape the biofilm. We also observed the differential expression of 19 genes carying putative small RNA molecules, indicating a novel role for these regulatory elements in chlorhexidine tolerance.

The high tolerance of sessile (biofilm-grown) *Burkholderia cepacia* complex bacteria (a group of 17 closely related bacteria) to antimicrobial agents (both antibiotics and disinfectants) is well established (23–26). This high tolerance limits the treatment options for infected cystic fibrosis (CF) patients (33) and complicates the implementation of effective infection control measures in hospitals and CF centers (28, 33). In addition, *B. cepacia* complex bacteria often are recovered as contaminants in various products (e.g., sterile pharmaceutical products) (13). However, information on the molecular mechanisms behind this tolerance still is limited. It was shown recently that the exposure of biofilms formed by *Burkholderia cenocepacia* strain J2315 to the oxidizing agents H_2O_2 and NaOCl results in an upregulation of the transcription of 315 (4.4%) and 386 (5.4%) genes, respectively (25). In addition, the expression of 185 (2.6%) and 331 (4.6%) genes was downregulated. Not surprisingly, many of the upregulated genes were involved in the general stress response and the response to oxidative stress or encode proteins involved in the repair of damage induced by reactive oxygen species (25).

Chlorhexidine is a biguanide disinfectant often used for skin disinfection, hand washing, and oral care (19). It compromises bacterial membrane integrity, resulting in the loss of periplasmic enzymes and cytoplasmic components (1, 14, 30), while at higher concentrations the coagulation of the bacterial cytoplasm can occur (11, 30). Very high levels of chlorhexidine resistance were described recently for several *B. cepacia* complex strains (including *B. cenocepacia* J2315) involved in outbreaks in CF patients, with MICs of >100 μg/ml (26). While there is no absolute correlation between chlorhexidine tolerance and the ability of bacteria to form biofilms (26), the increased resistance of biofilm-grown Gram-negative bacteria against chlorhexidine has been described previously (12, 21, 31). For example, Miyano et al. reported that 0.1 or 0.2% chlorhexidine gluconate had no effect on *B. cepacia* complex biofilms (21). In a separate study, Peeters et al. demonstrated that chlorhexidine treatments (0.015 or 0.05% for 15 min) failed to eradicate a substantial part of sessile *B. cenocepacia* populations, often resulting in regrowth after the discontinuation of the treatment (23). While strain- and model system-dependent differences make direct comparisons between different studies difficult at best, it is clear that biofilm-grown *B. cepacia* complex bacteria tend to show an increased tolerance toward chlorhexidine compared to that of planktonic cells.

In the present study, we determined the efficacy of chlorhexidine against planktonic and sessile populations of *B. cenocepacia* J2315 and determined the transcriptional response of biofilm-grown *B. cenocepacia* cells to chlorhexidine treatment (using microarray analysis and real-time quantitative PCR). In

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addition, the tolerance of mutant strains in which one or several efflux pumps were knocked out was evaluated.

MATERIALS AND METHODS

Strains and culture conditions. *B. cenocepacia* strain J2315 (LMG 16656) was obtained from the BCCM/LMG Bacteria Collection (Gent, Belgium). Mutant strains D1 (a resistance-nodulation-division-1 [RND-1] deletion mutant, ΔBCAS0591-0593), D3 (an RND-3 deletion mutant, ΔBCAL1672-1676), and D4 (an RND-4 deletion mutant, ΔBCAL2820-2822) have been described previously (3). All *B. cenocepacia* mutant strains were constructed by using methods described previously (5). The mutagenesis procedure allows for the creation of unmarked nonpolar gene deletions. Briefly, the upstream and downstream DNA sequence that flanks (about 500 bp each) the operon targeted for deletion were cloned into pGPI-SceI. This suicide plasmid contains a unique restriction site for the endonuclease I-SceI. The PCR amplifications of flanking regions for the construction of the mutagenesis plasmids were performed with the HotStar HiFidelity polymerase kit (Qiagen, Milano, Italy) by following the manufacturer's instructions. For the deletion of the RND-9 operon, the following primer pairs were used: (i) KO9XL (5′-TTTCTAGACGTACGTGCAGCAATGCC-3′; the XbaI site is underlined) and KO9BL (5′-TTGGATCCTCGACTACTACG AAGTGC-3′; the BamHI site is underlined) and (ii) KO9BR (5′-TTGGATCC TCAACCTCGACGAATGC-3′; the BamHI site is underlined) and KO9KR (5′-TTGGTACCAGCCTGTTCCTGACGAC-3′; the KpnI site is underlined). Mutagenesis plasmids were mobilized by conjugation into *B. cenocepacia* J2315 and the D4 mutant strain. Exconjugants were selected in the presence of trimethoprim (800 μg/ml), and the single-crossover insertion of the mutagenic plasmid in the *B. cenocepacia* genome was confirmed by PCR analysis. Subsequently, a second plasmid, pDAI-SceI (encoding the I-SceI endonuclease), was introduced by conjugation. Site-specific double-strand breaks take place in the chromosome at the I-SceI recognition site, resulting in tetracycline-resistant (due to the presence of pDAI-SceI) and trimethoprim-susceptible (indicating the loss of the integrated mutagenic plasmid) exconjugants. The desired gene deletions were confirmed first by PCR amplification and then by the Southern blot hybridization of XhoI-cleaved genomic DNA. In strain D9, the BCAM1945-1947 operon (RND-9) plus the transcriptional regulator BCAM1948 are knocked out, while the double mutant D4-D9 lacks both the RND-4 (BCAL2820-2822) and RND-9 operons. All strains were cultured on Luria-Bertani agar (LBA; Oxoid, Hampshire, United Kingdom) at 37°C.

Determination of efficacy of chlorhexidine treatment against planktonic and sessile cells. To assess the effect of chlorhexidine on planktonic cells, a modified European suspension test was used as described previously (23). Cells were treated with 0.0005, 0.015, and 0.05% chlorhexidine gluconate solution (ABC Chemicals, Woutersbrakel, Belgium) for 15 min. For biofilm formation, an inoculum suspension containing approximately 5×10^7 CFU/ml was prepared in Luria-Bertani both (LBB) starting from an overnight culture. Subsequently, 100 μl of this suspension was added to the wells of a round-bottomed polystyrene 96-well microtiter plate (TPP, Trasadingen, Switzerland). After 4 h of adhesion at 37°C, the supernatant (containing nonadhered cells) was removed and the wells were rinsed using 100 μl saline (0.9% NaCl); 100 μl of sterile LBB was added to all wells, and the biofilms were allowed to grow for an additional 20-h period. The supernatant was removed again, and 120 μl of a 0.0005, 0.015, or 0.05% chlorhexidine gluconate solution was added. Saline (120 μl) was added to biofilms that served as untreated controls. After 15 min, the chlorhexidine or saline was removed, and cells were removed from the wells and quantified by plating. All solutions were prepared using MQ water (Millipore, Billerica, MA) and were filter sterilized before use (Puradisk FP30; Whatman, Middlesex, United Kingdom). Experiments were carried out in duplicate or triplicate. Confidence intervals were calculated to determine whether survival was different between conditions and/or between strains (95% confidence interval, $P < 0.05$).

RNA extraction. Chlorhexidine-treated (0.015% for 15 min) and untreated *B. cenocepacia* J2315 sessile cells were harvested by vortexing (5 min) and sonication (5 min; Branson 3510; Branson Ultrasonics Corp., Danbury, CT) and transferred to sterile tubes. RNA was extracted immediately following the harvesting of the cells using the Ambion RiboPure bacteria kit (Ambion, Austin, TX) according to the manufacturer's instructions, and the procedure included a DNase I treatment of 1 h at 37°C. After extraction, the RNA was concentrated using Microcon YM-50 filter devices (Millipore).

Microarray hybridization and data analysis. The assessment of RNA yield and quality, cDNA synthesis, and the hybridization and washing of the custom-made 4 × 44K arrays (Agilent, Santa Clara, CA) was performed as described previously (25). The gene expression analysis was performed using GeneSpring GX 7.3 (Agilent), and data were normalized using the normalization procedure recommended for two-color Agilent microarrays. Only features dedicated to *B. cenocepacia* J2315 that were labeled present or marginal were included in the analysis. After these initial filter steps, an arbitrary cutoff of a 2-fold ratio change was applied to identify differentially expressed genes. Subsequently, a one-way analysis of variance (ANOVA) was performed ($P < 0.05$). The experimental protocols and the raw microarray data can be found in ArrayExpress under the accession number E-MEXP-2502.

RT-qPCR. To validate the microarray results, the expression of 29 genes in the chlorhexidine-treated biofilms (28 protein-coding genes and a single intergenic region) was examined by using reverse transcription-quantitative PCR (RT-qPCR) (Table 1). Biofilm formation, chlorhexidine treatment, RNA extraction, DNase I treatments, and cDNA synthesis were performed as described previously (25). Forward and reverse primers were developed using tools available on the NCBI website, and they were compared to the *B. cenocepacia* J2315 database using BLAST to determine their specificity. All qPCR experiments were performed on a Bio-Rad CFX96 real-time system C1000 thermal cycler as described previously (25). Each sample was spotted in duplicate, and an interrun calibrator as well as control samples without added cDNA were included in each experiment. The initial 3-min denaturation step at 95°C was followed by 40 amplification cycles consisting of 15 s at 95°C and 60 s at 60°C. A melting-curve analysis was included at the end of each run. To allow the accurate normalization of our data, we also included five reference genes (BCAL0421, BCAL1459, BCAL1861, BCAM0991, and BCAS0175) for which we confirmed expression stability prior to the actual analyses (see Fig. S1 in the supplemental material).

Evaluation of the fold change correlation between qPCR experiments and microarray analysis. For each gene examined by qPCR, the fold change in expression between the untreated and chlorhexidine-treated biofilms was compared to the corresponding fold change determined in the microarray analysis. To this end, the $\log_2$ fold changes were subjected to bivariate regression analysis, and Pearson and Kendall correlation coefficients were determined using SPSS 15 (SPSS Inc., Somers, NY).

Characterization of putative sRNA molecules. All intergenic regions for which a signal was obtained in the microarray analysis were considered to putatively contain a small RNA (sRNA) gene. To prevent the overestimation of the number of sRNA genes, candidate genes having expression levels similar to those of the neighboring genes were excluded from further analysis. Only intergenic regions were included (i) that were statistically upregulated at least 2-fold in treated versus untreated biofilms and (ii) for which the fold upregulation differed from that of at least one its flanking genes by at least a factor two. All predicted sRNAs were characterized as described previously (4). The secondary structure and the minimal free energy were calculated with Unafold software as implemented on the DINAMelt server (18). To assess whether secondary structure was more pronounced than expected for a sequence of identical length and composition, sequences were shuffled with *shuffleseq* (EMBOSS), and the free energy of 10 shuffled sequences was compared to that of the predicted sRNA using a one-sample *t* test (SPSS). To infer the function of the putative sRNAs, sequences were compared to sequences present in the RFAM database (8). The conservation of the putative *B. cenocepacia* J2315 sRNA genes in the genomes of other *Burkholderia* genomes was determined by BLASTn (35). Sequences were considered conserved if the alignment covered at least 50% of the query and the e-score was above 0.1.

Microarray data accession number. The experimental protocols and raw microarray data can be found in ArrayExpress under the accession number E-MEXP-2502.

RESULTS AND DISCUSSION

Effect of chlorhexidine on planktonic and sessile *B. cenocepacia* J2315 cells. Planktonic cultures contained $1.8 \times 10^9 \pm 1.5 \times 10^9$ CFU/ml (averages ± standard deviations), while sessile populations contained $4.6 \times 10^8 \pm 1.7 \times 10^8$ CFU/biofilm ($n = 6$). The treatment of planktonic and sessile cultures with increasing concentrations of chlorhexidine resulted in dose-dependent killing for both types of populations (Fig. 1). In low concentrations (0.0005%) chlorhexidine had a very limited bactericidal effect (killing less than 15% of cells in both populations), while a high concentration (0.05%) resulted in 96 and 95% killing for planktonic and sessile populations, respectively. However, at intermediate concentrations (0.015%) the bactericidal effect of chlorhexidine was much more pro-

TABLE 1. Primers used for qPCR

Gene	Primer sequence	
	Forward	Reverse
BCAL0421	GTTCCACTGCATCGCGACTT	GGGCTTCGTCGAATTCATCA
BCAL1110	AGCTGCCGTCCGACCTGTTC	TACGCGGCCATCGTGCAGAA
BCAL1459	ATCCCTTGAAATCGAGCATCA	TACGCTCGACAACGTGCATTA
BCAL1857	GCGGTGCCGATCCTTCCGTTTA	ACACGATCTCGCCGATCACGAC
BCAL1861	GATCACCTGCTTCGTGACGTT	GACGTCGTGTTCCGCAAGAT
BCAL2370	TGGAACGACGGCGACTGCAT	CGTGTGCTGGATGCCGGAGA
BCAL2451	ATCGCGATGGCGTGGAACATGA	AGAACAGCACGATGCCCAGGTC
BCAL2606	TCGACGACGACCAAGAACTC	GCAGCACGGACACTTCCATCC
BCAL2607	GAACCAGCCGATCCTCGACCTG	GCCGTGCTGACCATCGTCTTCT
BCAL2820	CTGCCATCGGCTGGCGCGAA	GCACCGATACGCGCAGGTCT
BCAL2821	GGGCCGCTGGCTCATCATCT	GCGTCTTCGCGGTCGTTTCC
BCAL2822	GCGGTGTTCCCGAACCCGAAT	GCTCGACCTTGTTGCTGGCGT
BCAL2823	ATGCGATGTTCGACCGCGTGTT	CCGCGACGTACTCGCACTTCAT
BCAL2846	TACCGCCTCCAAGGCCGATCAA	CGGTCAGGTCGCGTTCGGATTC
BCAM0772	ACCGCATCGCGAAGATCCTGA	CGAATGCGCTTCGGCCAGTG
BCAM0773	TGGGCGAACGACAGCACGAA	GCGACGCGTGGTAGCCCTT
BCAM0924	ACATCGCACGGCACACTCCA	GCGAGTACGTCCCAGCCGTT
BCAM0925	CGCTTCAGCAGCAACCGGTGA	GGCCGACGGAACAGCAGCT
BCAM0926	GACCGCGGTATTCCTGCCGA	ACGGCCATCGACAGCGTGAA
BCAM0928	TTGCGCCGGAGACGAAAGTGAC	GCGCCAGTAACCTCGCGAACAT
BCAM0991	GCCAACGTCTACCGGATGAA	GACCGTGCCGATGATGTAGAA
BCAM2073	CGCCGACTTCGACAAGGCCTAC	CCTGGCGTGCGACTTCCTTCAG
BCAM2537	CCAGATCGCGCTCGTGACGTTC	GCAACCCGAACACGCTGTCGAA
BCAS0081	ATGGGGCACGTGGTCGACTG	GCGCAACCGCATCGGAAAGT
BCAS0083	CGGCTTCCATGCGACGTCCAC	GGCTTCAGGCTCCCGTCGATC
BCAS0175	ATGGCCAGTTCGCTCATCA	ACGCGATGTCGATACTCGAAT
BCAS0637	ATGCGCGGCATCCTGAAGGT	CGAGCTGCTTGCCGGTTTCC
BCAS0638	CGGCGAGGAACTGCTCGTGA	TTTCCTGGCGGCCGGTGATT
IG_2538832	GCCGGCCGACGCGTTACAATA	GGCGCGGTGCGCAGGTAT

nounced for planktonic cells (78% killing) than for sessile cells (47% killing; $P < 0.05$) (Fig. 1).

Transcriptomic response to chlorhexidine: microarray data analysis and confirmation of expression trends with qPCR. Of the 7,153 protein-coding *B. cenocepacia* J2315 genes represented on the array, 469 (6.56%) were significantly overexpressed (fold change, >2; $P < 0.05$) in sessile cells in response to chlorhexidine treatment, while the expression of 257 protein-coding genes (3.59%) was significantly downregulated (fold change, <0.5; $P < 0.05$) (Table 2; also see Tables S1 and S2 in the supplemental material). In addition, significant differential expression was noted for 121 intergenic regions (91 upregulated and 30 downregulated) and for 23 tRNA genes (all upregulated). No differential expression of rRNA genes was observed (Table 2). A breakdown of differentially expressed protein-coding genes by functional category is presented in the supplemental material (see Table S3). The majority of overexpressed protein-coding genes with known function encoded periplasmic and exported proteins or lipoproteins ($n = 62$); were required for the structure and function of the inner membrane ($n = 42$); were involved in transport or binding ($n = 42$); had a regulatory function ($n = 32$); or were involved in chemotaxis and motility ($n = 20$). The majority of protein-coding genes downregulated in sessile *B. cenocepacia* J2315 cells treated with chlorhexidine were involved in transport or binding ($n = 34$) or had a regulatory function ($n = 27$)

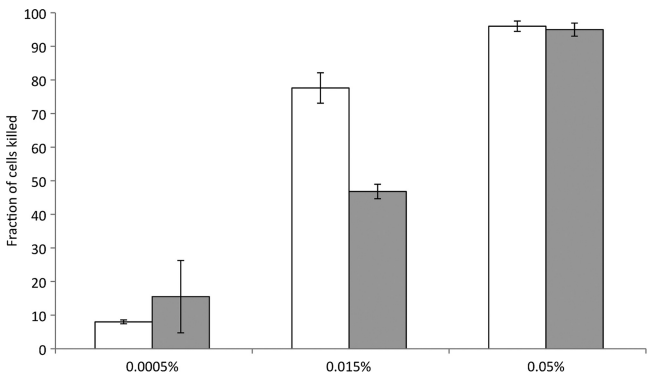

FIG. 1. Average fraction of cells killed by chlorhexidine treatment (0.0005, 0.015, and 0.05%; 15 min). White bars, planktonic cultures; gray bars, biofilm. Error bars represent 95% confidence intervals. The fraction of killed planktonic and sessile cells is significantly different for 0.015% chlorhexidine ($P < 0.05$).

TABLE 2. Proportion of genes significantly altering expression after exposure of sessile *B. cenocepacia* to chlorhexidine

Gene group (*n*)	No. (%) upregulated	No. (%) downregulated
CDS (7,153)	469 (6.56)	257 (3.59)
IG (1,520)	91 (5.99)	30 (1.97)
tRNA (47)	23 (48.94)	0 (0.00)
rRNA (9)	0 (0.00)	0 (0.00)
Total (8,729)	585 (6.70)	287 (3.29)

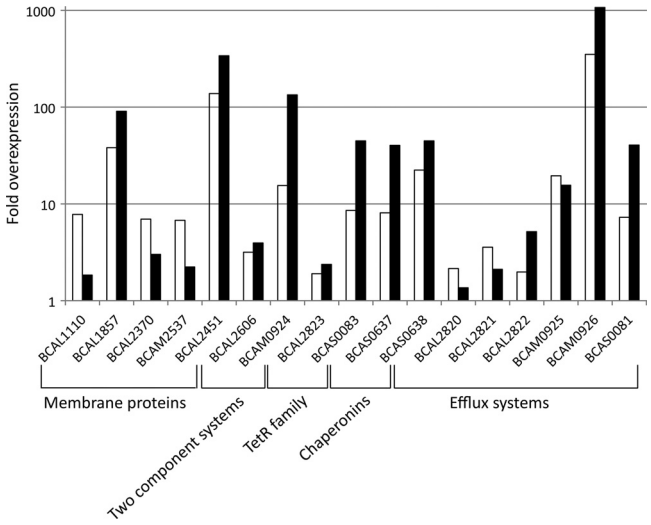

FIG. 2. Effect of treatment with chlorhexidine on the expression of a selection of protein-coding genes as assessed by microarray analysis (white bars) and qPCR (black bars).

(see Table S3). The expression level of 29 genes (28 protein-coding genes and 1 intergenic region) was confirmed by real-time qPCR. Overall, the expression data obtained with both approaches were similar (Fig. 2, Table 3), with highly significant ($P < 0.01$) Pearson and Kendall correlation coefficients ($r^2 = 0.480$ and $\tau = 0.652$, respectively).

Upregulation of putative drug resistance determinants. A wide range of potential drug resistance determinants was identified in the *B. cenocepacia* J2315 genome, including 16 efflux pumps belonging to the RND (resistance-nodulation-division) family and 10 efflux pumps belonging to the MFS (major facilitator superfamily) (9, 10). RND family efflux transporters function as a protein complex spanning the inner and outer membrane and are organized as tripartite efflux pumps consisting of a transporter in the inner membrane, a periplasmic accessory protein, and an outer membrane protein channel (9). Genes encoding these efflux systems typically are organized in an operon, although the outer membrane protein is not always included in the operon (9). The MFS is found in all living organisms and contains several families of secondary transporters, utilizing various transport mechanisms (6). Several of these potential drug resistance determinants were upregulated in sessile cells in response to chlorhexidine, including eight RND family efflux pumps and two MFS efflux transporters (Table 3).

The BCAM0925-0927 cluster encodes an RND family efflux transporter (RND-8) showing high similarity to the *adeABC* cluster in *Acinetobacter baumannii*, which is involved in aminoglycoside resistance (15). The expression of this operon could not be detected in planktonically grown cells (exponential phase, LB medium) (9) and basal expression in sessile cells in the absence of chlorhexidine was very low, explaining the very high upregulation of this operon following treatment (Fig. 2, Table 3). In *A. baumannii* the expression of AdeABC is regulated by the AdeRS two-component system (17), but no genes with significant similarity to *adeR* or *adeS* could be found in the *B. cenocepacia* J2315 genome. The RND-8 cluster is flanked by BCAM0924, coding for the response regulator protein of a two-component regulatory system, which also is markedly upregulated (Fig. 2). Whether this protein is involved in regulating the expression of the RND-8 efflux pump remains to be determined.

Two RND-type efflux systems (BCAL1674-1676 [RND-3] and BCAL2820-2822 [RND-4]) were shown recently to contribute to the high level of intrinsic resistance to several classes of antibiotics (3) and also were upregulated in sessile cells in response to chlorhexidine treatment. Remarkably, for most RND efflux systems, only one or two of the three genes showed significant overexpression, while the expression of the remaining partner(s) apparently was not altered by the chlorhexidine treatment. One of the MFS efflux transporters (BCAL3511,

TABLE 3. Differentially expressed potential drug resistance determinants

Drug resistance determinant	CDS	Fold overexpression by:	
		Microarray	qPCR
RND-3	BCAL1674	4.2	
	BCAL1675	1.5 (NS[a])	0.6
	BCAL1676	−1.2 (NS)	
RND-4	BCAL2820	2.2	1.4
	BCAL2821	3.6	2.1
	BCAL2822	2.0 (NS)	5.2
RND-6	BCAL1079	1.3 (NS)	
	BCAL1080	1.2 (NS)	
	BCAL1081	4.2	
RND-8	BCAM0925	19.5	15.6
	BCAM0926	351.0	1,070.4
	BCAM0927	100.7	3,601.0
RND-9	BCAM1945	2.2 (NS)	1.6
	BCAM1946	2.2	
	BCAM1947	1.6 (NS)	
RND-10	BCAM2549	2.0 (NS)	
	BCAM2550	−1.2 (NS)	
	BCAM2551	9.7	
RND-13	BCAL1811	4.1	
	BCAL1812	2.0 (NS)	
	BCAL1813	−1.0 (NS)	
RND-16	BCAM1419	−1.3 (NS)	
	BCAM1420	2.1	
	BCAM1421	2.8	
MFS efflux transporter	BCAL1510	1.1 (NS)	
	BCAL1511	1.4 (NS)	
	BCAL1512	2.4	
MFS efflux transporter	BCAL3511	2.0	
Aminoglycoside-3′-phosphotransferase	BCAM0928	3.9	
Fusaric acid resistance family transporter protein	BCAS0014	1.2 (NS)	
	BCAS0015	2.4	
	BCAS0016	−1.0 (NS)	
	BCAS0017	1.1 (NS)	

[a] NS, not significant.

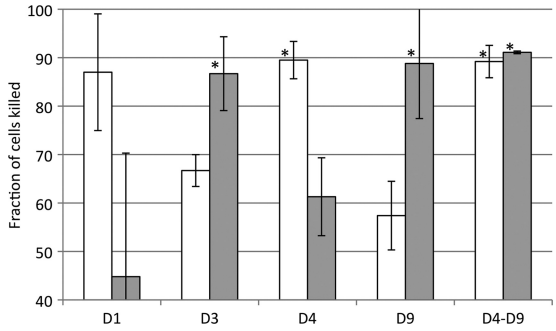

FIG. 3. Average fraction of cells of RND efflux pump mutants killed by chlorhexidine treatment (0.015%; 15 min). White bars, planktonic cultures; gray bars, biofilm. Error bars represent 95% confidence intervals. An asterisk indicates that the result is significantly different from that for the fraction of cells killed in wild-type cultures ($P < 0.05$).

also known as *bcrA*; 2.0-fold upregulated in response to chlorhexidine) was identified previously as an immunodominant efflux pump in *B. cenocepacia* J2315 and belongs to the 14 transmembrane domain subfamily (34). The overexpression of *bcrA* in *Escherichia coli* resulted in an increase in resistance to tetracycline and nalidixic acid (34); its apparent involvement in chlorhexidine efflux confirms its broad substrate range.

Identification of lifestyle-specific chlorhexidine tolerance mechanisms. To further investigate the role of several RND efflux pumps, we evaluated the susceptibility of planktonic and sessile *B. cenocepacia* RND mutants (Fig. 3). In the absence of chlorhexidine, the growth of the mutant strains was not affected and planktonic cultures as well as biofilms reached approximately the same density as the corresponding wild-type cultures (data not shown). Compared to results for wild-type cells, chlorhexidine tolerance was not altered in planktonic and sessile cells of the RND-1 mutant (D1), and this is in agreement with the lack of chlorhexidine-induced expression of the BCAS0591-0593 operon. However, when grown in a biofilm, strains D3 and D9 were less tolerant to chlorhexidine than wild-type cells, while the tolerance of planktonic cells was unaltered. In contrast, planktonic cells of strain D4 were more susceptible than wild-type planktonic cells, while the tolerance of sessile cells was not altered. The double mutant D4-D9 was hypersusceptible, both in planktonic and biofilm cultures, confirming the phenotypic observations for the single mutants. These data indicate that different RND efflux pumps are associated with chlorhexidine tolerance in planktonic and sessile cells, indicating the presence of lifestyle-specific chlorhexidine tolerance mechanisms. So far only a few biofilm-specific resistance mechanisms have been documented at the molecular level. In *P. aeruginosa*, the biofilm-specific synthesis (mediated by *ndvB*) of highly glycerol-phosphorylated cyclic-β-1,3-glucans that sequester antibiotics and interfere with their movement through the periplasmic space has been described (16, 27). A second example is the biofilm-specific expression of the PA1874-1877 efflux pump in *Pseudomonas aeruginosa* (36). The deletion of these genes results in increased sensitivity to several antibiotics, especially during biofilm growth (36). Data from the present study indicate that these lifestyle-specific mechanisms are more widespread than currently appreciated.

Downregulated genes. Besides an upregulation of expression of some efflux systems, many genes encoding transport-related proteins were downregulated as a consequence of chlorhexidine treatment. Two clusters with genes for two different ABC transporters (BCAL1742-1743 and BCAL3038-3039) were significantly downregulated, while six genes encoding MFS efflux pumps (BCAL0861, BCAL0950, BCAL2625, BCAM0465, BCAM0861, BCAM1152, and BCAM1289) also were significantly downregulated. The most repressed gene was BCAM0545 (87.0-fold downregulated), which is annotated as the IIA-like nitrogen-regulatory protein PtsN of the phosphotransferase system. In addition, many genes encoding regulatory proteins (belonging to various families, including the LuxR, MerR, ArsR, GntR, LysR, and TetR families) and three genes annotated as being involved in anaerobic respiration (BCAM1019, BCAM1020, and BCAM1676) also were downregulated (see Table S2 in the supplemental material).

Differential expression of genes involved in adhesion and motility. Another downregulated gene in chlorhexidine-exposed sessile cells was BCAM2143 (2.9-fold downregulated), encoding the 22-kDa adhesion protein AdhA (10). This gene is required for the binding of *B. cenocepacia* J2315 to cytokeratin 13 and for the efficient invasion of squamous epithelium (32). Twenty genes encoding proteins involved in chemotaxis and motility (functional class 1.1.1; see Table S3 in the supplemental material) were upregulated and included genes encoding the flagellar motor switch proteins FliG, FliN, and FliM (BCAL0524, 2.2-fold; BCAL3505, 3.0-fold; and BCAL3506, 2.4-fold), the M-ring protein FliF (BCAL0525, 3.3-fold), the flagellar basal body rod proteins FlgB, FlgF, and FlgG (BCAL0564, 3.1-fold; BCAL0568, 2.6-fold; and BCAL0569, 2.6-fold), as well as seven (putative) methyl-accepting chemotaxis proteins (upregulated between 2.2- and 8.5-fold; see Table S1). Interestingly, the majority of these upregulated chemotaxis- and motility-related genes also were found to be upregulated in sessile *B. cenocepacia* J2315 cells exposed to H_2O_2 or NaOCl (16/20 for the H_2O_2-treated cells and 9/20 for the NaOCl-treated cells) (25). In addition, another nine genes belonging to this functional class (motility: BCAL0140, BCAL0520, BCAL0523, BCAL 0565, BCAL0570, BCAL0576, BCAL3501, and BCAM1424; chemotaxis: BCAL0126) were more than 2-fold upregulated in treated biofilms, but this upregulation was not statistically significant (data not shown). The downregulation of the *adhA* gene in sessile cells exposed to chlorhexidine, combined with the upregulation of many genes required for motility, may mean that the cells are reverting to a planktonic life style, possibly in an attempt to escape their stressful environment. Further research will be required to confirm this hypothesis.

Expression stemming from intergenic regions: identification of putative small RNA genes. As mentioned above, significant upregulation was noted for 91 intergenic regions (Table 2). However, a detailed comparison of the expression data for those intergenic regions with those of their neighboring coding sequences (CDS) revealed that these expression patterns were similar (suggesting cotranscription) for many intergenic regions. In addition, intergenic regions for which the probe(s) overlapped with coding sequences on the other strand also were excluded. Using these exclusion criteria, 19 intergenic regions were retained for further analysis (Table 4). The over-

TABLE 4. Intergenic regions for which the expression pattern differs from that of the neighboring CDS

Intergenic region	Fold change	Location (fold change)	Conservation[a]	Free energy (kcal/mol)
IG1_772886	2.2	BCAL0710 (−1.2)–BCAL0711 (−1.1)	*B. cepacia* complex	−113.9[c]
IG1_899543	2.2	BCAL0827 (1.3)–BCAL0828 (1.1)	*B. cenocepacia*, *B. lata*	−45.6[c]
IG1_901672	9.1	BCAL0830 (1.5)–BCAL0831 (1.6)	*B. cenocepacia*, *B. lata*, *B. multivorans*	−163.6[c]
IG1_1345312	4.5	BCAL1231 (1.0)–BCAL1232 (1.7)	*B. cenocepacia*, *B. lata*	−46.1[c]
IG1_1928097	3.5	BCAL1746 (1.6)–BCAL1747a (1.6)	*B. cenocepacia*	−75.9
IG1_2099347	3.6	BCAL1901 (1.0)–BCAL1902 (−1.1)	*B. cepacia* complex	−52.4[c]
IG1_2538832	2.1[d]	BCAL2285 (−1.2)–BCAL2286 (−1.7)	*B. cepacia* complex	−44.0[c]
IG1_2617451	7.8	BCAL2363 (2.1)–BCAL2364 (1.0)	*B. cepacia* complex (except *B. vietnamiensis*)	−76.9
IG1_3008003	8.6	BCAL2737 (1.2)–BCAL2738 (4.8)	*Burkholderia* genus	−274.1[c]
IG1_3556424	2.8	BCAL3236 (−250.0)–BCAL3237 (1.5)	*B. cenocepacia* J2315 and MC0-3, *B. lata*	−73.4[c]
IG1_3851358	2.3	BCAL3513 (−1.1)–BCAL3514 (1.3)	*B. cepacia* complex	−121.8[c]
IG2_208795	2.2	BCAM0180 (1.4)–BCAM0181 (1.0)	*B. cenocepacia* J2315, HI2424, and AU1054	−163.7[c]
IG2_1745287	4.2	BCAM1568 (−1.2)–BCAM1569 (1.2)	*B. cepacia* complex (except *B. lata*)[b]	−179.1[c]
IG2_1885142	3.1	BCAM1690 (1.4)–BCAM1691 (1.7)	*B. cepacia* complex	−41.9[c]
IG2_1975919	4.7	BCAM1760 (1.2)–BCAM1761 (2.6)	*B. cepacia* complex	−128.0
IG2_2085682	3.6	BCAM1866 (1.1)–BCAM1867 (1.5)	*B. cepacia* complex	−141.1[c]
IG2_2103360	3.1	BCAM1887 (1.3)–BCAM1888 (2.2)	*B. cenocepacia* J2315	−7.9
IG2_2718925	2.4	BCAM2414 (−1.4)–BCAM2415 (2.2)	*B. cepacia* complex	−123.2[c]
IG3_93582	3.0	BCAS0083 (8.6)–BCAS0084 (1.2)	*B. cenocepacia*	−38.6

[a] Conservation means that the sequence was retrieved from all genomes of that particular taxon, unless explicitly mentioned otherwise.
[b] A significant hit ($e = 2 \times 10^{-35}$, 91% query coverage) with *Entamoeba dispar* SAW760 hypothetical protein EDI_314460 was observed.
[c] Significantly lower free energy than the average free energy of 10 shuffled sequences of the same length and same composition ($P < 0.005$).
[d] This intergenic region also was investigated with qPCR and showed an overexpression of 1.2-fold.

expression of these 19 putative sRNA genes upon treatment with chlorhexidine ranged from 2.1 to 9.1. The majority (18/19) of these genes were not conserved outside the *B. cepacia* complex (the exception being IG1_3008003, which is found in all *Burkholderia* species for which a genome sequence is available). All but three intergenic regions (IG1_3556424, IG2_208795, and IG2_2103360) are conserved in all *B. cenocepacia* genomes. A single gene (IG2_2103360) appears to be specific for *B. cenocepacia* J2315. The folding free energy for the majority of the predicted structures is lower than that for shuffled sequences (Table 4), indicating a tendency toward a stable secondary structure (2). However, none of the putative sRNAs identified shows a significant match to the sequences in the RFAM database, and their function remains elusive.

Sigma factors. The *B. cenocepacia* J2315 genome contains 20 σ^{70} genes, of which 14 belong to the extracytoplasmic function (ECF) group (20). It also contains RpoN (BCAL0813, σ^{54}), which is involved in biofilm formation (29). In a previous study, we identified four sigma factors as being upregulated in response to oxidative stress (BCAL0787, BCAL1369, BCAL1688, and BCAL3478) (25). In the present study, overexpression was observed for BCAL2360 (EcfD; 2.6-fold upregulated), BCAL2462 (EcfE; 2.4-fold upregulated), and BCAL2872 (RpoE; 2.0-fold upregulated). Downregulation was observed for two sigma factors: BCAL3152 (putative sigma factor, 2.2-fold downregulated) and BCAL0813 (RpoN, 3.4-fold downregulated). The overexpression of the RpoE homolog BCAL2872 may be related to the overexpression of several RND efflux pumps. In *P. aeruginosa*, the induction of the MexCD-OprJ efflux system (which contributes to chlorhexidine resistance; see below for details) is dependent on the RpoE homolog AlgU (7), and the same may be true for *B. cenocepacia* J2315. The biological relevance of the downregulation of two sigma factors is unclear and requires further investigation.

Comparative transcriptomics: comparing the responses of *B. cenocepacia* J2315 and *P. aeruginosa* PAO1. Nde et al. (22) recently investigated the transcriptomic response of planktonically grown *P. aeruginosa* cells treated with 0.0004% chlorhexidine for 10 min. Resistance mechanisms identified included efflux and the upregulation of *oprH* to block chlorhexidine translocation by self-promoted uptake. In addition, the repression of aerobic cellular respiration (through the downregulation of genes involved in oxidative phosphorylation and electron transport) likely decreases energy production, contributing to chlorhexidine-induced growth inhibition. The expression of genes associated with a switch to anaerobic metabolism also was altered (e.g., upregulation of *narG*, downregulation of *aspA*). Although the experimental conditions used were different from the ones used in the present study, *P. aeruginosa* and *B. cenocepacia* often share the same ecological niches, and comparing both datasets may lead to novel insights into chlorhexidine tolerance. Our analyses indicated that several of the genes involved in chlorhexidine tolerance in *P. aeruginosa* (including *oprH* and *narG*) are absent from the *B. cenocepacia* J2315 genome. For the differentially expressed *P. aeruginosa* genes for which *B. cenocepacia* homologs could be identified, several patterns were observed. The upregulation of a common set of efflux pumps in response to chlorhexidine exposure was observed. Genes belonging to the *P. aeruginosa* PA4597-4599 cluster (encoding the MexCD-OprJ efflux pump) were significantly upregulated after chlorhexidine treatment. These genes code for an efflux pump of the RND family and share modular similarity to both the BCAM0925-0927 cluster (RND-8) and the BCAL2820-2822 (RND-4) cluster in *B. cenocepacia* J2315. A set of 19 *P. aeruginosa* genes was downregulated after 10 min of chlorhexidine treatment, but the expression of their homologs was unaltered in treated sessile *B. cenocepacia* J2315 cells. This set included genes encoding ribosomal proteins (S20 and L34) as well as several genes en-

coding proteins involved in central metabolism (pyruvate dehydrogenase, isocitrate dehydrogenase, and succinyl-coenzyme A synthetase). A final group contains genes that are repressed in treated planktonic *P. aeruginosa* cells but are upregulated in treated sessile *B. cenocepacia* J2315 cells. This group includes two genes involved in biotin metabolism (PA4847 [homolog, BCAL3420] and PA4848 [BCAL3421]) and PA5557 and PA5558, encoding δ- and β-subunits of the F_0F_1 ATP synthase complex. The latter genes were approximately 2-fold downregulated in *P. aeruginosa* but where 3.0- and 4.1-fold upregulated in *B. cenocepacia* J2315. These genes are part of a cluster of nine genes (PA5553-5561; *atpCDGAHFEBI*) coding for proteins that make up the F_0F_1 ATP synthase complex that synthesizes ATP from ADP in the presence of a proton gradient across the membrane. The same cluster can be found in the *B. cenocepacia* J2315 genome (BCAL0029-0037), but in contrast to *P. aeruginosa*, chlorhexidine-treated *B. cenocepacia* cells increase the expression of most genes in this cluster. Whether the increased expression of these genes relates to an increased need for ATP and/or is a response to cell damage remains to be explored.

Conclusions. Sessile *B. cenocepacia* J2315 cells are more tolerant to intermediate concentrations of chlorhexidine (0.015%) than their planktonic counterparts. The expression of many genes is affected when biofilm-grown *B. cenocepacia* J2315 cells are treated with chlorhexidine. Several genes encoding membrane-related and regulatory proteins, as well as several genes coding for drug resistance determinants (including RND and MFS efflux systems), were upregulated. The phenotypic analysis of RND efflux pump mutants confirms the presence of lifestyle-specific chlorhexidine tolerance mechanisms, with some efflux pumps being responsible for chlorhexidine efflux in planktonic cells and others being responsible for chlorhexidine efflux in sessile cells. The downregulation of a gene encoding an adhesin and the upregulation of many genes encoding chemotaxis and motility-related proteins indicate that sessile cells try to escape from the biofilm. The differential expression of putative sRNA genes located in intergenic regions suggests that these sRNA molecules also play a role in chlorhexidine tolerance. Finally, comparing our data with transcriptomic data obtained from planktonic *P. aeruginosa* cells indicates that there are both common and species-specific mechanisms for chlorhexidine tolerance.

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